Isolation and Sequences Analysis of Tight Junction Protein Claudin Encoding Genes in Intestinal Barrier of Common Carp (*Cyprinus carpio*)

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Abstract

Hamdan Syakuri and Dieter Steinhagen. 2014. Isolation and Sequences Analysis of Tight Junction Protein Claudin Encoding Genes in Intestinal Barrier of Common Carp (*Cyprinus carpio*). *Aquacultura Indonesiana, 15 (2) : 74-85.* Claudins multigene family encode proteins which forms main structures of tight junction among epithelial cells. In intestinal epithelium claudin proteins are proposed to have roles in ion balances regulation and forming barrier against pathogenic invasion. This work was done in order to identify claudin encoding genes in common carp (*Cyprinus carpio*), one of the most important cultured fish in freshwater aquaculture. Sample of RNA was isolated from the fish and transcribed into cDNA. Carp claudin genes were amplified, cloned, and sequenced. Carp claudin 1 and 2 were amplified with primers designed based on relevant known claudin genes in fish. On the basis of carp ESTs, other six carp claudin genes were amplified by using appropriate primers. Phylogenetic and sequence analysis confirmed that the eight identified genes are claudin genes and could be designated as carp claudin 1, 2, 3^b, 3^c, 7, 11, 23, and 30, respectively. This finding could be used to develop further study of carp claudin especially roles of claudin during pathogen infection and strategy to modulate its expression in order to protect diseases in carp aquaculture.

Keywords: Claudin; Cyprinus carpio; Intestinal barrier

Introduction

Common carp (Cyprinus carpio) is considered as one of the most important commodities of aquaculture, especially in freshwater fish culture in Asian and some European countries (FAO 2011). Common carp is not classified as a high-priced food; however, it is becoming an essential protein source in human diets. In the last two decades the annual production of common carp increased exponentially and reached more than 3 millions tones in 2010 (FAO 2011). Currently, it represents 14% of the global freshwater aquaculture production and is mainly cultivated in Asian countries especially in China which accounts for 70% of the world total production (FAO 2011). The development of common carp industry is challenged by many factors including protection against pathogenic invaders. With respect to this challenge, intestinal barrier functions have moved into the focus of research.

As the first barrier against external invaders from the lumen of the gut, the intestinal epithelium performs a broad range of innate defense mechanisms. The most outer layer, the mucus acts as physical and chemical barrier that protects the epithelium from microbial adherence and colonization, and this layer also might contain antimicrobial substances (Turner 2009). Another physical barrier is formed by the epithelial cells and their apical intercellular junctional structures, the tight junctions (TJs) which prevent microbial internalization (Lal-Nag and Morin, 2009). A major component of TJs is claudin proteins which are not only responsible for allowing ion and water transport but also for building the intestinal physical barrier against foreign antigens (Morita et al., 1999; Lal-Nag and Morin 2009). According to many reports, claudin proteins are regulated during diseases or inflammation processes, and in some cases they targets of pathogens (Berkes et al., become 2003; Prasad et al., 2005; Groschwitz and Hogan 2009; Guttman and Finlay 2009; Schulzke et al., 2009).

Claudin is a multiple genes family. A total of 24 claudin genes were reported from mammals and in particular humans and chimpanzees lack claudin-13 (Lal-Nag and Morin 2009). Fish claudins were at the first time identified in zebrafish, *Danio rerio* (Kollmar *et al.*, 2001) and then found in many fish species such as Japanese pufferfish, *Takifugu rubripes* (Loh *et al.* 2004); spotted green pufferfish,

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Tetraodon nigroviridis (Bagherie-Lachidan *et al.*, 2008); Atlantic salmon, *Salmo salar* (Tipsmark *et al.*, 2008^a); southern flounder, *Paralichthys lethostigma* (Tipsmark *et al.*, 2008^b); rainbow trout, *Onchorhynchus mykiss* (Chasiotis and Kelly, 2011; Sandbichler *et al.*, 2011); and goldfish, *Carassius auratus* (Chasiotis and Kelly, 2011). However knowledge on fish claudin is still limited. The present work was done in order to molecularly identify claudin protein encoding genes in an important species in freshwater aquaculture, common carp (*Cyprinus carpio* L.).

Materials and Methods

Sampling of Intestinal Tissues and RNA Extraction

After fish was killed, intestinal pieces were collected and placed directly in 1.5 mLtubes filled with 1 mL of RNA isolation reagents TriZol (Life Technologies) and then stored at -80°C until further use. RNA was extracted according to protocol from the manufacturer. Tissue samples were ground using a Tissue lyser (Oiagen). The resulting suspension was transferred to a new 1.5-mL tube and incubated for 5 minutes at room temperature. After separation with 0.2 mL chloroform addition, RNA was precipitated from upper aqueous phase with 0.5 mL of isoprophanol. The RNA pellet was washed twice with 1 mL of 75% ethanol and then was dissolved with RNase-free water and heated at 55°C for 10 minutes. Finally, quantity and purity of the samples were determined in a Nanodrop spectrophotometer and then the samples were kept at -80°C until use. Samples with a ratio of absorbance A_{260/280} around 2.00 and $A_{260/230} = 2.00-2.20$ were accepted as pure RNA samples.

Digestion of Genomic DNA and Construction of cDNA

Prior to the construction of cDNA a possible contamination with genomic DNA was

removed by using RNase-free DNase I (Fermentas) according to manufacturer's protocol. Briefly, a mix of RNA (1 μ g), 1 μ L of 10x buffer with MgCl₂, 2 units of DNase, and 10 units of ribolock (RNase inhibitor) in DEPC-treated water (up to 10 μ L of final volume) was incubated for 30 minutes at 37°C. Subsequently, the DNase was inactivated by adding 1 μ L of 25 mm EDTA and incubation at 65°C for 10 minutes.

The cDNA was constructed using Maxima Reverse Transcriptase (Fermentas) as described in the manufacturer's protocol. RNA samples were mixed with a mix of 0.25 µL oligo dT (100 pmol) and 0.25 µL random hexamer (100 pmol), 1 µL dNTPs (10 mm each), 4 µL of 5x RT buffer, 0.5 µL ribolock (20 u), 1 µL Maxima Reverse Transcriptase (200 u) and nuclease-free water (until a total volume of 20 µL). The mixture was incubated for 10 minutes at 25°C followed by 30 minutes at 50°C. At the end, the reaction was terminated by heating at 85°C for 5 minutes. Then the cDNA samples were preserved at -20°C until further analysis. Amplification of β -actin by using primers which cover intron on few non reverse transcribed samples confirmed genomic DNA no contamination.

Primers Design

Primers for carp claudin 1 and 2 were designed based on gene specific sequences of other fish species and primers for carp claudin 3^b, 3^c, 7, 11, 23, and 30^c were designed on the basis of relevant expressed sequences tags (ESTs) of carp available in gene bank (see Table 1). The primers were designed using the software Primer3 (Rozen and Skaletsky, 1999) and the melting temperature of the primers was calculated using the software of Integrated DNA technologies OligoAnalyzer 3.1 available at eu.idtdna.com. All primers used in the present study are listed in **Error! Reference source not found.**

Gene	Name	Sequence ($5 \rightarrow 3$)	Product size(bp)
Claudin-1	Cyca_cl1F	tgccaggagaggttcagg	147
	Cyca_cl1R	cagccacagccactttattc	
Claudin-2	Cyca_cl2F	gctctggagttgatgggtttc	189
	Cyca_cl2R	gtaggtctcgcattggaagg	
Claudin-3 ^b	Cyca_cl3BF	atatgggagggaatctggatg	288
	Cyca_cl3BR	gtgagcagaccagcatacag	
Claudin-3 ^c	Cyca_cl3CF	tcacggcacaagtcatctgg	342
	Cyca_cl3CR	cggtggacagtaaccgggttg	
Claudin-7	Cyca_cl7F	ccccaatggaagatgtctgc	547
	Cyca_cl7R	aaacgtactccttgctgctg	
Claudin-11	Cyca_cl11F	cttatcatcgccactgccac	416
	Cyca_cl11R	tacagggagaagccaaaggac	
Claudin-23	Cyca_cl23F	agggaatctgggacatctgc	290
	Cyca_cl23R	gctggtgaggatatgagtgtacc	
Claudin-30 ^c	Cyca_cl30F	atcggcagcaacatcgtcac	350
	Cyca_cl30R	aacagagggttgtagaagtcc	
β-actin	BF	ggtatgggacagaaggacagc	300
	BR	ggcatacagggacagcac	

Table 1. Primers used for gene amplification

Claudin Genes Amplification

For PCR, the standard reaction mix consisted of 1X PCR buffer (Invitrogen), 0.2 mm of each dNTPs (Fermentas), 1.5 mm of MgCl₂ (Invitrogen), 0.2 µm of each primer, 1 unit of Platinum Taq DNA polymerase (Invitrogen), 2 µL of template (cDNA), and in total volume of 20 µL. The PCR assays were performed in a Mastercycler gradient (Eppendorf). The cycle's program was: initial denaturation, 95°C, 10 min.; denaturation, 95°C, 30 s.; annealing, 55-59°C, 45 s.; extension, 72°C, 30 s.; 40 cycles; and an additional extension, 72°C, 5 min. Amplification products were confirmed by electrophoresis on ethidium bromide or GelRed pre stained 1.5% agarose gels and visualized with UV light. The rest of the amplification products were kept at -20°C until further use.

Cloning of The Genes

Amplification products of each gene were cloned into the pGEM-T easy vector (Promega) according to the manufacture's protocol. Briefly, 2 μ L of PCR product was mixed with 1 μ L of ligase (3 U/ μ L), 0.5 μ L vector (25 ng), and 5 μ L of 5x ligation buffer in a total volume of 10 μ L. After incubation at 4°C over night, 2 μ L of the ligation product was mixed with 25 μ L of *Escherichia coli* JM109 High-Efficiency Competent Cells (Promega) and placed on ice for 20 minutes. The mix then was heat-shocked at 42°C for 45 seconds and placed back on ice for 2 minutes. After adding of 970 μ L of LBB and incubated at 37°C for 2 hours, 50 μ L of the concentrated bacterial suspension was poured on LB/ampicillin/IPTG/X-gal plates. White colonies were collected, cultured in LBB for 16-18 hours and then the recombinant plasmid was isolated.

The recombinant plasmid was isolated the GeneJet plasmid miniprep kit using (Fermentas) according to the protocol from the Briefly, pelleted manufacturer. cells were resuspended in 250 µL of resuspension solution and lysed using 250 µL of lysis solution. After the process was terminated using 350 µL of neutralization solution. the samples were centrifugated at more than 12000 x g for 5 minutes and the supernatant was transferred to a separation column. After two washing steps, the plasmid samples were eluted and stored at -20°C until further use.

The presence of a DNA insert in the recombinant plasmid was confirmed by an endonuclease digestion assay. Four μ L of every plasmid were mixed with 0.5 μ L of *Eco*R1 and 1 μ L of buffer in a total volume of 10 μ L. After incubation at 37°C for 2 hours, the DNA plasmid and the DNA insert were separated on a 1.5% agarose gel pre stained with GelRed (Biotium) in 100 mV for 30 minutes and visualized under UV light.

Sequencing and Sequence Analysis

Recombinant plasmids were directly sequenced (LGC Genomicy GmbH, Germany). Nucleotide sequences of carp claudin encoding genes were confirmed as part of targeted genes using a BLASTn analysis (Altschul *et al.*, 1990)

and translated in silico into an amino acid sequence with a DNA translation tool available online at www.expasy.org (Gasteiger et al., 2003). Further confirmation of carp claudins was performed based on local and global models Pfam HMMs as motif sources by MyHits motif scan analysis (Pagni et al., 2004; Pagni et al., available at www.myhits.isb-sib.ch. 2007) Transmembrane domains of claudins were predicted by SOSUI (Hirokawa et al., 1998; Mitaku and Hirokawa 1999; Mitaku et al., 2002) available at bp.nuap.nagoya-u.ac.jp. Protein motifs of carp claudins were compared with those of relevant animals by means of multiple sequence alignment analysis using ClustalW (Larkin et al., 2007) in default settings (www.ebi.ac.uk). The Phylogenetic relationship of carp claudin genes with appropriate genes of other fish were analyzed by following steps and alignment, MUSCLE; methods: curation. Gblock; phylogeny, phyML; and tree rendering, treeDyn at www.phylogeny.fr (Guindon and Gascuel 2003; Edgar 2004; Anisimova and Gascuel 2006; Dereeper et al., 2008; Dereeper et al., 2010).

Results

From common carp intestinal tissues, partial fragments of eight carp claudin encoding genes were successfully amplified, cloned, sequenced, and confirmed as parts of claudin encoding genes by using BLASTn analysis (www.ncbi.nlm.nih.gov) and/or by motif scan analysis (www.myhits.isb-sib.ch). Two claudin genes, claudin-1 and -2 were amplified from cDNA of carp by using primers designed on the basis of the same genes of other animals. Sequences of the other six claudin genes of carp: claudin-3^b, -3^c, -7, -11, -23, and -30 were identified by using primers designed on the basis of claudin-like ESTs from carp, which were obtained by performing BLASTn analysis of all zebrafish and pufferfish claudins on the EST database available in the GenBank.

A phylogenetic tree, constructed on the basis of the nucleotide sequences of claudins and shown in Figure 1, describes the relationship of the eight carp claudin genes from this study with other claudin genes identified in teleost species. According to the consensus phylogenetic tree of claudin proteins (Loh *et al.*, 2004), six carp claudin genes were assigned to the first gene cluster and two carp claudin genes were assigned to the second gene cluster. On the basis of the

phylogenetic analysis and according to the claudin nomenclature proposed for Japanese pufferfish claudins (Loh *et al.*, 2004), names for carp claudins were considered. In cases when only one isoform was identified, the carp claudin gene was named according to the main name of the pufferfish claudin and not to the name for an isoform. Six carp claudin genes sequences were deposited in the GenBank (accession number JQ767156- JQ767160). Only fragments of less than 200 bp were obtained from claudin-1 and claudin-2 and therefore the sequences of these two genes could not be deposited in the GenBank because of an internal policy of this data base.

Typical molecular properties of the multiple genes family of tight junction protein claudins include two cytoplasmic tails, four transmembrane (TM) domains, one intracellular loop (IL), two extracellular loops (ELs), and two highly conserved cysteine residues in the first extracellular part (Lal-Nag and Morin 2009). In order to confirm identified genes in this work, partial protein sequences of carp claudin genes were in silico transcribed, their molecular motifs were predicted, and the level of identity among other fish claudins was analysed. A fragment of carp claudin-1 consists of an incomplete domain of TM-2, IL, and few amino acid residues of TM-3 (Figure 2). Parts of TM-1 and the extracellular loop 1 (EL-1) of carp claudin-2 were identified (Figure 3). The identified fragment of carp claudin-3b showed parts of EL-1, TM-2, IL, and TM-3 (Figure 4). Features of EL-1, TM-2, IL, TM-3, and EL-2 were determined from a partial protein sequence of claudin-3c (Figure 5). All properties of claudin-7 except of the amino-tail, TM-1, and few residues of EL-1 were identified in carp (Figure 6). A fragment that is starting from EL-1 until carboxy-tail of carp claudin-11 was reported (Figure 7). Fragments consisting of EL-1, TM-2, IL, and TM-3 were identified as parts of carp claudin-23 (Figure 8), and claudin-30 (Figure 9). In comparison to the same genes of other fish species, claudin genes of carp showed different level of identity. For examples; carp claudin-30 was 93% identical to claudin-b of goldfish (Figure 9) and carp claudin-11 was 92% similar with claudin-11^a of zebrafish (Figure 7). In contrast, carp claudin-1 was only 63% and 60% identical with the genes of zebrafish and Japanese pufferfish, respectively (Figure 2) and the sequence of carp claudin-3^b had a low identity to claudin-3^b of the Japanese pufferfish or claudin-3 of Atlantic salmon with 62% and 67%, respectively (Figure 4).



Figure. 1. Unrooted cladogram of fish claudin genes.

The phylogenetic analysis included carp claudin-1, -2, -3^b (JQ767156), -3^c (JQ767157), -7 (JQ767155), -11 (JQ767158), -23 (JQ767159), and -30 (JQ767160) encoding genes, and its relevant genes from pufferfish (*Takifugu rubripes*), zebrafish (*Danio rerio*), Atlantic salmon (*Salmo salar*), and tilapia (*Oreochromis mosambicus*). The carp claudin gene sequences were obtained from this study and the other sequences were retrieved from GenBank. The accession numbers are mentioned in the leaves names. The carp claudin genes are indicated by the arrows.

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	TM-1		EL-1	\vee	
ZebrafishCldn1	MAHAGLQMLGYCLGFLGLLGLIASTAMAE	WKMSSYAGD	NIITAQAQY	EGLWQSCVSQST	G 60
PufferfishCldn1	MANAGIQLLGFILAFLGLIGTIASTIMVE	WKASSYAGD	NIITAQAMY	EGLWKSCVSQST	G 60
CarpCldn1					-
	\forall	TM-2		IL	
ZebrafishCldn1	QLQCKKYDSLLKLPGEIQGA RGLMLTGIF	LCGLSTLVS	FVGMKCTTC	LSEAPOVKSKVA	L 120
PufferfishCldn1	QIQCKVYDSLLQLPGIVQGTRGLMLASVL	LSVICLLVE	MVGMRCTTF	MAEEPEQKDKVA	L 120
CarpCldn1		GVGLLVA	AVGMKCTTC	LSDEKEOKNKVA	V 29
		: **	***:***	:::: : *.***	:
	TM-3	EL-2		TM-4	
ZebrafishCldn1	AGGVLFITGGLFALIATSWYGEKIRQKFF	DPFTPTNAR	TEFGKALYV	GWGSSALSIIGG	S 180
PufferfishCldn1	AGGVIFIIAGLLALVGTSWYGHRIAREFY	DPFTPTNSR	YEFGSALYV	GWGAACLTLIGG	G 180
CarpCldn1	AGGV				- 33

ZebrafishCldn1	LLCCICGSEASEK-PSYPPARAAGRPGTE	DRV 210			
PufferfishCldn1	FLCCSCPKKGSOKSPRYPPTRSSGPOGKE	YV 211			
CarpCldn1					A
)),					

R	SeqA: Name (length)	SeqB: Name (length)	Score
D	CarpCldn1 (33 aa)	ZebrafishCldn1 (210 aa)	63.0
	CarpCldn1 (33 aa)	PufferfishCldn1 (211 aa)	60.0
	ZebrafishCldn1 (210 aa)	PufferfishCldn1 (211 aa)	65.0

Figure 2. Multiple alignment of deduced protein sequences of fish claudin-1 genes (A) and their pairwise scores (B). The analysis included claudin-1 genes of common carp (*Cyprinus carpio*; present work), zebrafish (*Danio rerio*; accession number: NM_131770), and pufferfish (*Takifugu rubripes*; accession number: AY554352). Domains of four transmembranes (TM, bold letters), two extracellular loops (EL), and an intracellular loop (IL) are indicated. Arrows show two highly conserved cysteine residues.

Carp Zebr Puff Carp Zebr Puff	Cldn2 afishCldn2 erfishCldn2 cldn2 afishCldn2 erfishCldn2	PCGLI MAILALELMGFFFGL MASAALELMGFFFGL ↓ ↓ ↓ AFQCETYNTLLGLTVI AFQCETYNTLLGLTVI AFQCETYNSMLALPSI	TN-1 PGMLGTFV IGMLGTLV FGLLGTHV .*.***.* DLQAARAM DMQASRAL	ATLLPYWEM ATLLPYWAT: STVLPYWQI: ;*;**** TM-2 MVMSSIFSVI MVISLVLSI:	SAHVGSNIV SAHVGPNIV SAHIGSNIV ***:*.*** MACAVSTVG LAIALAVLG	EL-1 EAVESMKGI EAVVSMKGI EAVGNMRGI EL MQCTVCMDC MQCTVCMDC	V WIECVYQSTG MMECVYQSTG 	49 60 60 119 120
		<u>TM-3</u>		EL-	2	TM-4	L	
Carp Zebr Puff	Cldn2 afishCldn2 erfishCldn2	VGGSMFLLAGLLSLI VGGGLFLTAGFLSLI	PVAWKTHE PVAWTTHE	VVQTFYMPNI VVQTFYRPD	MPASLKFEI LPSSLKFEL	BCLYVGL	ASSLLEMLGGG	179 180
Carp Zebr Puff	oCldn2 afishCldn2 erfishCldn2	LLSASCCDDIDGNRG	SRRHYPYP NGRGYPYP	ERNALRGPS LGANVRT	hsmtyqpaai Isqtyrnpti	LHSTTNPN	TNKTQTLNSH NRGRAVVRTI	239 238
Carp Zebi Puff	cldn2 afishCldn2 erfishCldn2	TSSGTHSIQAQDSRK SDSSHPSLHGNQGGK	MTRONTAA KPAAA	GYDVTGYV GYDITGYV	270 266		,	4
-	SeqA: Name (length)	SeqB: Name	Score					
B	CarpCldn2 (49 aa)	ZebrafishCldn2 (270 aa)	83.0					
	CarpCldn2 (49 aa)	PufferfishCldn2 (266 aa)	75.0					
	ZebrafishCldn2 (270 aa)	PutferfishCldn2 (266 aa)	56.0					

Figure 3. Multiple alignment of deduced protein sequences of fish claudin-2 genes (A) and their pairwise scores (B). The analysis included claudin-2 genes of common carp (*Cyprinus carpio*; present work), zebrafish (*Danio rerio*; accession number: NM_001004559), and pufferfish (*Takifugu rubripes*; accession number: AY554353). Domains of four transmembranes (TM, bold letters), two extracellular loops (EL), and an intracellular loop (IL) are indicated. Arrows show two highly conserved cysteine residues.

			TM-	1	EL-1	\vee	
Puff	erfishCldn3b	MASVGLEILGVELGV	LOWILAIVS	CIVPMWRVSAFIGMN.	IVTAQITWEGIW	INCVVQSTG	60
ACIA	inticualmoncidns	MAVLGUBILGMILAV	LOWILSIVE	CALPHWRVSAFIGVN	111AQTIMEDIW	HCVVQS10	60
CP66	2816mix	MAALGLETLGVTLAV	FOWIVGIVS	CALPHWEVTAFTGVN	TVTAOTTWEGTW	INCVVQSTG	60
	A C C C MARL				a range a nora ni		
		\vee		TM-2	IL		
Puff	erfishCldn3b	QMQCKVHDSMLALSA	DLQAARALT	IISIVLGVMOIVVAM	MGSKCTNCVGED	SAKARVMIA	120
Atla	inticsalmonCldn3	QMQCKVYDSMLALSS	DLQAARALT	VISIVVGIMGVLVAV	VGAKCTNCVKDE	TAKARVMIA	120
CREE	Clonso Calemix	QMQCK11DSMLALSS	DLOVARALC	UTATUMOVLOFLLST	VGIECTECLERE	CVEARVMIT	120
	A G & G MARK	*****, ********	***.**.*	*********	1*.***.*		
		TM-3		EL-2	TM-	4	
Puff	erfishCldn3b	AGVAFLLAALTQLVP	VSWSAHHII	MEFYNPMIPETQKRE	IGAALYLOWAAA	AFLLIGGGV	180
Atla	anticsalmonCldn3	AGVAFIVASLTQLIP	VSWSANSII	MEFYSPITPEAHKRE	IGGALYLGWAAAJ	AFLLIGGCI	180
CR66	Cidnab California	AGVIPICAAVMHLI-	WWWWWWW	POPUDDITICLORPE	TOTELVIOWACE	ALIDIROOG	82
100	A DADBER	***1*1 *11 1*1	(Charles a L	tor for an any set		An of the owner of the	
Puff	erfishCldn3b	LVSSCPKPPAKRYG-	- PSNITYPQ	SRSMVPSVYDRRDYV	217		
Atla	inticsalmonCldn3	LCCSCPPQPEKRYAG	SPSRMVYSL	TRSVAPSGYDKRDYV	219	3	
Cart	Cldn3D Setemin	PCAAAA, DDCDDTC		TUDIORETILU	205	- F	4
	VEDIOUITY	CONNU-LEGERIG-		- I VBERGWE I LULR-	200		
D	SeqA: Name (length)	SeqB: Name (length)	Score				
B	CarpCldn3b (82 aa)	CF662816mix (205 aa)	89.0				
	CarpCldn3b (82 aa)	PufferfishCldn3b (217 aa)	62.0				
	CarpCldn3b (82 aa)	AtlanticsalmonCldn3 (219 aa)	67.0				
	CF662816mix (205 aa)	PufferfishCldn3b (217 aa)	59.0				
	CF662816mix (205 aa)	AtlanticsalmonCldn3 (219 aa)	63.0				

Figure 4. Multiple alignment of deduced protein sequences of fish claudin-3^b genes (A) and their pairwise scores (B). The analysis included carp EST (CF662816; a mix library of skeletal white muscle, cardiac muscle, brain, gill, kidney and intestinal mucosa tissues) and claudin-3^b genes of common carp (*Cyprinus carpio*; present work; accession number: JQ767156), Atlantic salmon (*Salmo salar*; accession number: BT048428) and pufferfish (*Takifugu rubripes*; accession number: AY554378). Domains of four transmembrane (TM, bold letters), two extracellular loops (EL), and an intracellular loop (IL) are indicated. Arrows show two highly conserved cysteine residues.

PufferfishCldn3b (217 aa) AtlanticsalmonCldn3 (219 aa) 73.0

PufferfishCldn3C (216 aa) ZebrafishCldnh (214 aa)

			TM-1	EL-1	Y		
Carp	Cldn3c			EGLV	MNCVVQSTGQ	14	
EC39	3103skin	MSMGLELGGIALGIIG	VIISIVTCALPMWRVSAFV	GANIVTAQVIWEGL	MNCVVQSTGQ	60	
Zebr	afishCldnh	MSMGLEIGGIALGIIG	WIISIVACALPMWRVSAFV	GANIVTAQVIWEGLU	MNCVVQSTGQ	60	
Puff	erfishCldn3C	MSMGMEIVGIALGVLG	FIITIVSTALPMWRVTAFI	GANIITAQTIWEGLV	MNCVTQSTGQ	60	
Carr	Cldn3c	MOCKTYDEMLALCODI	TM-2	IL ISIMGAKCTNCIEDE	CKAKUMTUS	74	
ECIE	3103gkin	MOCKIYDSMLALSODL	ASPAMSVVSTTLATLOVL	TSIMGAKCTNCIEDE	CASKAKUMTUS	120	
Zebr	afishCldnh	MOCKVYDSMLALGODL	DASRAMTVIAIILAVLOVM	ISVMGAKCTNCIEDE	GAKAKVMIVS	120	
Puff	erfishCldn3C	MOCKVYDSLLALTPEL	DASRAMMITATILGVLGVM	ISTVGAKCTNCIEEE	GAKAKVMITA	120	
		****:***:***	****** 111***.1***1	**::*******	* * * * * * : :		
		TM-3	EL-2	TM-	.4		
Carp	Cldn3c	GVMFIIAGILELIPVA	VANQTILVF			100	
EC3S	3103skin	GVMFIIAGILELIPVA	WANQTIRDFYNPLLSTAQ	QRELGASIYIGFAAJ	ALLIGGAML	. 180	
Zebr	afishCldnh	GIMFIIAGILDLIPSAWVANQIIRDFYNPLLPGAQQRELGASIYIGFAAAALLIIGGAML					
Puff	erfishCldn3C	GIFFILGGVLVLIPVS	WTAHVVIRDFYNFILNNSQ *.*: * *	RRELGAALYIGWAAN	ALLLIGGAML	. 180	
(1. m. m.							
ECSC	3103akin	CCTCPPPPEKKYKPPPPM	TYEAPPEVERG V	- 208		12	
Zebr	afishCldnh	CCTCPPKEKKYKPARM	JYSAPRSASAG YDKKDY	V 214		A	
Puff	erfishCldn3C	CSSCPPKEEKYKPPRM	AYSTPRSASAGGVYDRKDY	V 216			
D	SeqA: Name (length)	SeqB: Name (length)	Score				
в	CarpCldn3c (100 aa)	EC393103skin (208 aa)	98.0				
	CarpCldn3c (100 aa)	PufferfishCldn3C (216 aa)	68.0				
	CarpCldn3c (100 aa)	ZebrafishCldnh (214 aa)	84.0				
	EC393103skin (208 aa)	PufferfishCldn3C (216 aa)	71.0				
	EC393103skin (208 aa)	ZebrafishCldnh (214 aa)	88.0				

Figure 5. Multiple alignment of deduced protein sequences of fish claudin-3^c genes (A) and their pairwise scores (B). The analysis included carp EST (EC393103, skin library) and claudin-3^c genes of carp (*Cyprinus carpio*; present work; accession number: JQ767157), zebrafish (*Danio rerio*; accession number: NM_131767), and pufferfish (*Takifugu rubripes*; accession number: AY554367). Domains of Four transmembrane (TM, bold letters), two extracellular loops (EL), and an intracellular loop (IL) are indicated. Arrows show two highly conserved cysteine residues.

75.0

Carp EC39 Zebr	Cldn7 3792skin afish7b	TM-1 MANKGLQLLGFTLSLL MAHKGLQLLGFTLSLL	GLIGLIVGI GLIGLIIGI	ILPQWKMSAYVGD	EL-1 NIITAIAMYQGI NIITAIAMYQGI NIITAIAMYQGI	V LWMSCAFQSTG LWMSCAFQSTG LWMSCAYQSTG	28 60
Puff	erfishCldn7b	MANSGMQLLGFFMBLI	GIVALIIGI	ILPQWKMSAYIGD	NIITAVAMYQGI	WMSCAFQSTG	60
Carp EC39 Zebr Puff	Cldn7 3792#kin afish7b erfishCldn7b	QMQCKVYDSILQLDSS QMQCKVYDSILQLDSS QQQCKVYDSVLQLDSS QLQCKIYDSILQLDSS	LQATRALMI LQATRALMI LQATRALMI LQATRALMI	TM-Z VGILLTVAGLGVA VGILLTVAGLGVA VAILLTVAGLGVA VGIIMSIAGLGVA	SMGMKCTNCGGI SMGMKCTNCGGI SMGMKCTNCGGI CMGMKCTTCGG	DEKVKKSRIAM DEKVKKSRIAM DEKVKKSRIAM IDKLRKSRVAM	88 120 120 120
Carp EC39 Zebr Puff	Cldn7 37928kin afish7b erfishCldn7b	TM-3 TGGIVLIVGALCSIVA TGGIVLIVGALCSIVA TGGIILSVGALCSIVA TGGIILLVGGLCAIVA	CGWFINQIV CGWFINQIV CGWFISQII CSWFAHNVI	EL-2 RDFYNPFTPVNTK RDFYNPFTPVNTK RDFYNPFTPVNTK RAFYNPYTPVNSK	YEFGAAIFIAW YEFGAAIFIAW YEFGAAIFIAW YEFGAAIFIAW FEFGBAIFIAW	TM-4 AGAFLDIMGGG AGAFLDIMGGG AGAFLDIMGGG 3GSLLDVLGGA	148 180 180 180
Carp BC39 Zebr Puff	Cldn7 3792skin afish7b erfishCldn7b	MLAASCPKGKSSPRYP MLAASCPKGKSSPRYP MLASSCSKGQSSPNYP MLAASCPRNKHLPKYP	KSSR KSSR KSSRPVH PTASSR ***	PPQ PPSS5KEYV SSRPPS55KEYV -SGAPS5TKEYV .*.	171 209 215 213		4
-	SeqA: Name (length)	SeqB: Name (length)	Score				
в	CarpCldn7 (171 aa)	EC393792skin (209 aa)	99.0				
	CarpCldn7 (171 aa)	PufferfishCldn7b (213 aa)	72.0				
	CarpCldn7 (171 aa)	Zebrafish7b (215 aa)	90.0				
	EC393792skin (209 aa)	PufferfishCldn7b (213 aa)	74.0				
	EC393792skin (209 aa)	Zebrafish7b (215 aa)	91.0				
	PufferfishCldn7b (213 aa)	Zebrafish7b (215 aa)	69.0				

Figure 6. Multiple alignment of deduced protein sequences of fish claudin-7 genes (A) and their pairwise scores (B). The analysis included carp EST (EC393792, skin library) and claudin-7 genes of common carp (*Cyprinus carpio*; present work; accession number: JQ767155), zebrafish (*Danio rerio*; accession number: NM_131637), and pufferfish (*Takifugu rubripes*; accession number: AY554347). Domains of four transmembrane (TM, bold letters), two extracellular loops (EL), and an intracellular loop (IL) are indicated. Arrows show two highly conserved cysteine residues.

Carp DW72 Zebr Puff	Cldnl1 2473testis afishCldnl1a erfishCldnl1a	TM- MANTCLQFTGFVMBFLG MANTCLQFTGFVMBFLG MANSCLQLGGFLVSCLG	1 WIGLIIA WIGLIVA	V EL- EWVVTCKYNN TATNEWVVTCKYNN TATNEWVPTCKYOI TSTNEWVNMCKYGI	1 V MTCKRMDELEV MTCKRMDELEV MTCRKMDELEAR MTCRRMDELEAR	GLWADCVIST GLWADCVIST GLWADCVIST GPWAECVIST	32 60 60 60
Carp DW72 Zebr Puff	Cldnii 2473testis afishCldniia erfishCldniia	TM-2 ALYHCITLTQILELPAY ALYHCITLTQILELPAY ALYHCITLTQILELPAY GLYHCVSLTQILDLPAY	L TQTSRAL TQTSRAL TQTSRAL TQTTRAL	TM-3 MVTASILGLPAVAI MVTASILGLPAVAI MVTASILGLPAVVA MITGSILGFVAVVA	VLMSMSCINLGS VLMSMSCINLGS VLMSMSCINLGG KLLMSMPCISLGN	EL-2 EPESAKNKRS EPESAKNKRS EPESAKNKRS IEAQGSKNRRA	92 120 120 120
Carp DW72 Zebr Puff	Cldnii 2473testis afishCldniia erfishCldniia	TM-4 VLGGTIILLTAPCGVVS VLOGTIMLLTAPCOIVS VLGGIIILLTAPCOIVS VLG-RSAGSHRLCSLVS *** :**	TVWFPIG TVWFPIG TVWFPIG	AHHEKGIMSF AHHERGIMSFGFSI AHHEKGIMSFGFSI AHQQBGIMSF GFSI	LYNONE LYSGWVGTALCLI LYAGWVGTVFSLI	GGCIISCCSV GGSLLTCCSS	126 163 180 179
Carp DW72 Zebr Puff	Cldnll 2473testis afishCldnlla erfishCldnlla	D-SPATYTENNRFYYSK DSLPRSFQDNNRFYYSK	QGPAHTG QGGGNAF	PT-STNHAKSAHV ATSATNHAKSAHV	215 216		A
D	SeqA: Name (length)	SeqB: Name (length)	Score				
D	CarpCldn11 (126 as)	DW722473tests (163 aa)	96.0				
	CarpCidn11 (126 aa)	PufferfishCldn1ta (216 aa)	65.0				
	CarpCldn11 (126 aa)	ZebrafishCidn11a (215 aa)	92.0				
	DW722473tests (163 as)	PutterfishCkdn11a (216 aa)	65.0				
	DW722473testis (163 aa)	ZebrafishCldn11a (215 aa)	89.0				
	PufferfishCldn11a (216 aa)	ZebrafishCkln11a (215 aa)	66.0				

Figure 7. Multiple alignment of deduced protein sequences of fish claudin-11 genes (A) and their pairwise scores (B).

The analysis included carp EST (DW722473, testis library) and claudin-11 genes of common carp (*Cyprinus carpio*; present work; accession number JQ767158), zebrafish (*Danio rerio*; accession number: NM_001002624), and pufferfish (*Takifugu rubripes*; accession number: AY554358). Domains of four transmembrane (TM, bold letters), two extracellular loops (EL), and an intracellular loop (IL) are indicated. Arrows show two highly conserved cysteine residues.

			TM-1		$\mathbf{E}(\mathbf{I}_i - \mathbf{I}_i)$	¥		
Car	pCldn23					TS	2	
CA9	64745m1x	MRTPGILIFC	MVLAPCG	WILDLTSTVAPNWRTIN	NLANEPSDL	VVEQGIWDICRTS	56	
Puf	ferfishCldn23a	MRTSLRTPGILIFO	LVLSPCG	WVLNLTATVAPNWRTLH	NIFGIQADA	FLQQGIWDICRAT	6.0	
		504.92				11		
		\vee		TM-2		IL		
Car	pCldn23	TISRSQLCNQQGNI	QIYFNSQ:	IIPIAKGMMVASLIVTL	LGLALATEG	VRCWKDRPRWILA	62	
CA9	64745m1x	TTSRSQLCNQQGNI	QIYFNSQ:	IIPIAKGMMVASLIVTL	LGLALATPO	VRCWRDRPRWILA	116	
Puf	ferfishCldn23a	STESRAECGLQ I	TTYFNHE:	IIPVAQGLMVASLVVTI	IGLIVAIPG	VRCWRDNPNWTVA	118	
		2*. *. * *	*** ::	***:*:*:*****:**:	.** .* **	****:*.*.* :*		
		<u>TM-3</u>		EL-2		TM-4		
Car	pCldn23	SLOGLLIFCSGAL	TIPIA				81	
CA9	64745mix	SLGGLLIFCSGAL	TIPIAWY	THILTSINSTSVKRDPN	RADDIRVGY	CIILGYIGGIMEV	176	
Puf	ferfishCldn23a	GLGGLLIFISGVF	TIPISWY	THLINEIPSSS	PLTDVEVGY	CIVLOFIGGIFEI	172	

Car	pCldn23							
CA9	64745mix	LGGFVMFLGIFSCO	GGRIRGE	KSRTYTTQRPA	T		209	
Puf	ferfishCldn23a	LAGFVMFIGICRCO	GGKNRGEI	RRVEEVMEERYKQQKPE	PRRFEVPSL	NRARSSFESSVPY	232	
Car	nCldn23							
CAS	64745mix						A	
Puf	ferfishCldn23a	CENCE DEVEDEARCENTETUCCEPUTADE, 267						
			the second second		×			
B	SeqA: Name (length)	SeqB: Name (length)	Score					
D	CarpCldn23 (81 aa)	CA964745mix (209 aa)	100.0					
	CarpCldn23 (81 aa)	PufferfishCldn23a (267 aa)	53.0					
	CA964745mix (209 an)	Putterfieh/3de/23e /267 as)	56.0					

Figure 8. Multiple alignment of deduced protein sequences of fish claudin-23 genes (A) and their pairwise scores (B). The analysis included carp EST (CA964745, a mix library of skeletal white muscle, cardiac muscle, brain, gill, kidney and intestinal mucosa tissues) and claudin-23 genes of common carp (*Cyprinus carpio*; present work; accession number: JQ767159), and pufferfish (*Takifugu rubripes*; accession number: AY554348). Domains of four transmembrane (TM, bold letters), two extracellular loops (EL), and an intracellular loop (IL) are indicated. Arrows show two highly conserved cysteine residues.

			TM-1	EL-1 V			
Car	pCldn30			QTSWEGIWMSCVVQST	0 17		
EC394291skin		MVSAGLQLLGTALAIIGWIGVIVVCAIPMWKVTAFIGSNIVTALTSWEGIWMSCVVQSTG					
601	dfishCldnb		GWIGVVVSCALPMWKVTAF	IGNNIVTAQTSWEGIWMTCVVQST	G 44		
Zeb	rafishCldnb	MASTGLOMLGIALAI	GWIGVIVLCALPMWKVTAF	IGANIVTSQTSWEGIWMSCVVQST	G 60		
Puf	ferfishCldn30c	MVSTGFQMLGAALGIV	GWIGAIIACALPMWKVTAF	IGSSIITAGTTWEGIWMNCVVQST	G 60		
		W.	TM-2	TT.			
Car	pCldn10	CMOCKVYDEMLALSSI	LOAARALTIIAIVIGILGI	MLAMAGGRETICIEDESSKSKVGV	T 77		
EC3	94291skin	OMOCKVYDEMLALSEI	LQAARALTIIAIVIGILGI	MLVHAGGKCTNCIEDESSKSKVGV	T 120		
Gol	dfishCldnb	QMQCKVYDSMLALSSI	LQAARALTIISIVIGILGI	MLAMAGGKCTTCIEDESSKSKVGI	T 104		
Zeb	rafishCldnb	QMQCKVYDSMLALSSI	DIQAARALTVISIVIGVMGI	MLSMAGGKCTNCIEEESSKAKVG1	T 120		
Puf	ferfishCldn30c	QMQCKVYDSMLALGBI	LQAARALVIISIMVGIIGI	LLAVAGGECTNCVEDPTAKAEVGI	A 120		
					*		
(TAY)	0606100	SOUTPITACULCUTP	Corrana TV-	18-9	- 101		
EC3	94291skin	SGVIFILAGVLCLIP	CWTAHVIVODFYNPLLNOA	OKRELGAALYIGWGAAALLIIGGG	L 180		
Gol	dfishCldnb	AGVIFIIAGVLCLIP	CWTANVIVODFYNPLLNOA	OKRELGAALFIGWGAAALLIIGGG	L 164		
Zeb	rafishCldnb	AGVIFIISGVLCLVP	CWTANAI IQDFYNFLVVQA	QEREIGASLYIGWGASALLIIGGS	L 180		
Puf	ferfishCldn30c	AGVAFIVAGILCLIPT	CWIAHVVVRDFNNFLVPAA	MKRELGAALYIGWGAAALMLIGGA	L 180		
			****:-::				
Car	oCldn30						
EC3	94291skin	LCCNCPPEDDTGEYT	KYSAPPRSAASAPSGKNF-	214			
Gol	dflehCldnb	LCCQC		169			
Zeb	rafishCldnb	LCCHCPEKSDSGKYTZ	AKYNATPREEASAPSOKNFV	215			
pur	ferfiancidn30c	LCCNCPKK-DKGSYT	ARYNAARSKASAPASGKDYV	214	A		
	SeqA: Name (length)	SeqB: Name (length)	Score				
в	CarpCldn30 (101 aa)	EC394291skin (214 aa)	96.0				
	CarpCldn30 (101 aa)	PufferfishCldn30c (214 aa)	75.0				
	CarpCldn30 (101 aa)	GoldfishCldnb (169 aa)	93.0				
	CarpCldn30 (101 aa)	ZebrafshCldnb (215 aa)	85.0				
	EC394291skin (214 aa)	PutferfishCldn30c (214 aa)	71.0				
	EC394291skin (214 aa)	GoldfishCldnb (169 aa)	91.0				
	EC394291skin (214 aa)	ZebrafshCldnb (215 aa)	82.0				
	PufferfishCldn30c (214 aa)	GoldfishCldnb (169 aa)	76.0				
	PufferfishCldn30c (214 aa)	ZebrafishCidnb (215 as)	68.0				
	GoldfishCldnb (169 aa)	ZebrafishCidnb (215 aa)	85.0				

Figure 9. Multiple alignment of deduced protein sequences of fish claudin-30 genes (A) and their pairwise scores (B). The analysis included carp EST (EC394291, skin library) and claudin-30 genes of common carp (*Cyprinus carpio*; present work; accession number: JQ767160), zebrafish (*Danio rerio*; accession number: AF359426), Gold fish (Carassius auratus; accession number: HQ656008) and pufferfish (*Takifugu rubripes*; accession number: AY554369). Domains of four transmembrane (TM, bold letters), two extracellular loops (EL), and an intracellular loop (IL) are indicated. Arrows show two highly conserved cysteine residues.

Discussion

Correspond to the current state of typical profiles of members in claudin multiple genes family (Lal-Nag and Morin, 2009) fish claudins genes are characterized by four transmembrane helices and two extracellular loops (ELs). The two extracellular structures of claudins allow them to build intercellular junctional structures which mainly involve the second EL and mediate selectively barrier permeability functions of TJs that are mainly facilitated by the first EL (Van Itallie and Anderson, 2004, 2006). Roles of the first extracellular domain in size and charge paracellular selectivity have been at least described in claudin-2, -4 (Colegio et al., 2003), -5 (Wen et al., 2004), and -7 (Alexandre et al., 2007). Furthermore two conserved cysteine residues that were predicted to form protein intramolecular disulfide bond for stabilization (Wen et al., 2004; Angelow et al., 2008; Lal-Nag and Morin 2009) are consistently present in EL-1 domains of fish claudins.

In piscine studies, the first identification of claudin genes was made in Zebrafish (Kollmar et al., 2001). Thereafter, molecules from the claudin gene family were found in other fishes (Loh et al., 2004; Bagherie-Lachidan et al., 2008; Tipsmark et al., 2008^a; Tipsmark et al., 2008^b; Chasiotis and Kelly 2011; Sandbichler et al., 2011). Interestingly, in the Japanese pufferfish the claudin gene family comprises a higher number of members (56) than in mammals and some of them represent fish specific claudin genes (Loh et al., 2004). In the present study, eight claudin genes, claudin-1, -2, -3^b, -3^c, -7, -11, -23, and -30, were identified from common carp. In gene expression studies, transcript level of some claudin genes in common carp was modulated under Koi Herpesvirus (KHV) infection (Adamek et al., 2013, Syakuri et al., 2013) and after feeding beta-glucan and Aeromonas hydrophila intubation (Syakuri et al., 2014). Therefore further studies are needed and modulation of the genes expression could be considered as a part of diseases protection strategies in common carp cultivation.

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